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(71) Applicant: IMMUNICON CORPORATION [US/US]; Suite 1310, Mansons Mill Business Campus, Huntingdon Valley, PA 19006 (US).

(72) Inventor: LIBERTI, Paul, A. ; 58 Green Drive, Churchville, PA 18966 (US).

(74) Agent: MCMORROW, Robert, G., Jr.; Dann, Dorfman, Herrell and Skillman, 1310 The Fidelity Building, 123 S. Broad Street, Philadelphia, PA 19109 (US).

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(54) Title: DIRECT HOMOGENEOUS ASSAY

(57) Abstract

Method and test kit for determining the presence of a bindable substance, e.g. antigen or antibody, in a test sample in excess of a predetermined amount and the quantitative measurement of any such excess.

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DIRECT HOMOGENEOUS ASSAY

FIELD OF THE INVENTION

The present invention is directed to the qualitative and quantitative determination of specific binding substances. The invention enables the presence or amount of one substance of any specific binding pair of substances to be determined by means of its complementary binding substance. In particular, the present invention is useful for determining whether a specific binding substance is present in a test sample in excess of a predetermined amount. The invention also permits quantitative measurement of any excess of the specific binding substance present in the test sample above a predetermined amount. Although the present invention has a variety of applications, it is particularly useful in the determination of immunochemical binding substances in biological fluids, for example, the detection or quantification of antigen or antibodies in human serum.

PRIOR ART

A number of different types of immunoassays are currently in use for the determination of the components of immunochemical binding pairs, e.g. antigens and antibodies, in various biological fluids. In general, an immunoassay, involves the determination of an immunoreactive substance, either directly or indirectly, by means of an immune reaction between the immunoreactive substance and a labeled form of its immunospecific conjugate or other receptor system.

Even substances that are not immunogenic by themselves, such as haptens, can be determined by immunoassay if they are bound to larger carrier substances which are capable of inducing antibody to the lower molecular weight substance. Immunoassays are useful in the detection of immune reactions in blood serum and are also employed in a number of immunohistochemical methods performed on tissue.

Immunoassays may be carried out with all of the immunochemical binding substances in solution, or with the immunoreactive substance or its immunospecific conjugate affixed to a solid support, such as glass or plastic tubes, beads, or microtiter plates. The latter is known as a solid-phase immunoassay. Solid-phase immunoassay is widely used due to its simplicity of performance and the ease with which the labeled complex may be separated from the unreacted, labeled antigen or antibody.

One type of solid-phase immunoassay involves a direct non-competitive binding technique for determination of immunochemical binding substances. In performing an immunoassay of this type to determine antibody, for example, antigen is immobilized on a solid support and then contacted with the test sample containing the antibody to be determined. Thereafter, a second antibody which is labeled and reacts specifically with the first antibody is added to the test sample. Because the second antibody is labeled the presence of the first antibody in the sample may be determined. By means of this technique, commonly known as a "sandwich technique", specific antibody can readily be detected in blood serum.

Solid phase immunoassays are also commonly

performed as competitive binding assays, based upon competition between labeled and an unlabeled forms of an immunoreactive substance for binding sites on the immunospecific conjugate. In order to determine the amount of antigen present in a test sample using a competitive assay, the test sample is usually mixed with labeled antigen, and then contacted with the corresponding antibody bound to a solid support, with the labeled and unlabeled antigen competing for antibody binding sites. The test sample is then separated into a liquid phase and a solid phase and the relative amount of labeled antigen present in either phase is quantitatively determined.

Most competitive binding immunoassays operate on the principle that an immunoreactive substance present in a test sample and a labeled form of the same substance, are attracted with essentially equal affinity to a solid support bearing the immunospecific conjugate. Therefore, the labeled and unlabeled forms of the immunoreactive substances become linked to the support in amounts proportional to the relative amounts of each substance in the sample. For example, if a test sample were to contain 90% of unlabeled antigen and 10% of labeled antigen, and the two substances were permitted to compete for a limited number of binding sites on the corresponding insolubilized antibody, the ratio of unlabeled antigen to labeled antigen becoming bound to the antibody would be 9:1.

It is also known to conduct solid-phase immunoassays by the saturation technique, in which a portion of immobilized immunospecific conjugate is saturated with the immunoreactive substance being determined in the test sample, and thereafter the

remaining unbound immunospecific conjugate is bound to a quantity of the labeled form of the immunoreactive substance being detected.

The direct, competitive and saturation techniques used in performing solid-phase immunoassays each permit quantitative determinations of immunoreactive substances by way of calculations based on the extent of binding of the labeled reagent employed in the assay.

Radioisotopes, enzymes and various chromophoric substances are commonly employed as labels in the above-described immunoassays. Radioisotopes provide a readily measurable signal which permits the results of the assay to be determined directly. Immunoassays employing such labels are generally characterized by exceptional sensitivity and accuracy.

Enzymes have been proposed as labels for immunochemical binding substances, especially in assays intended for home use. A notable advantage of the enzyme labels is that enzyme activity is detectable by the naked eye or by inexpensive detection equipment, such as a colorimeter. In performing an immunoassay using an enzyme-labeled reagent, the enzyme activity is measured by using a suitable substrate for producing an enzyme-catalyzed reaction, typically involving production or extinction of a colored compound whose light adsorption may be readily determined either qualitatively or quantitatively. Immunoassays employing enzyme labels are commonly referred to as enzyme-linked immunoabsorbant assays (ELISA).

It is known in the prior art to implement the ELISA procedure using a so called "test strip" or "dip stick" technique. These assays employ a bibulous

substrate or carrier, which may be a natural or synthetic polymeric material, but is typically a cellulosic material, such as filter paper, to which is affixed the immunospecific conjugate of the immunoreactive substance being determined. The enzyme label may be linked to the immunospecific conjugate bound to the support, or with a component of the immunochemical binding pair in the test sample. These assays are intended to eliminate the manipulative steps, e.g. washing and incubation which are normally involved in the standard ELISA technique, thereby reducing the chance of error, so as to permit use by untrained personnel. Moreover, because the procedure may be conducted rapidly and gives a visual result, these assays are desirable for use in a doctor's office and in the home.

An ELISA-type assay employing a test strip having antibodies bound thereto is described in U.S. Patent No. 4,168,146. This assay is based on the aforementioned "sandwich technique". In use, the antibody-bearing test strip is immersed in the test sample suspected of containing the antigen to be determined. After the test sample migrates along the test strip, and any antigen present in the test sample reacts with the antibodies bound to the test strip, the test strip is contacted with a solution containing antibodies linked to an enzyme label and the enzyme activity is determined, as described above.

Another test strip-type assay, employing a combination of enzymes, is disclosed in U.S. Patent No. 4,435,504. The test strip is in the form of an immunochromatograph having a porous support permitting solvent travel, a plurality of a specific binding pair

substance (e.g. antibody) and an enzyme, the latter two components being non-diffusively and uniformly bound to the support to define an immunosorbing zone. Also employed in this assay is an enzyme-labeled specific binding pair member (e.g. enzyme-labeled antigen), which binds to the immunosorbing zone in relation to the portion of the immunosorbing zone in which the target substance being determined (e.g. antigen) is bound, to define a border related to the amount of target substance present in the test sample. It is disclosed that the enzyme label is preferably related to the enzyme on the immunochromatograph, such that the substrate of one is the product of the other. In practice, the immunochromatograph is contacted with the test sample for a time sufficient for the test sample to migrate across the immunosorbing zone. The immunochromatograph is also contacted with the labeled specific binding pair substance in a solvent medium, so that the labeled specific binding pair substance binds in the immunosorbing zone in relation to the target substance bound thereon. Thereafter, the immunosorbing zone is contacted with a development solution containing a substrate for the enzyme label to produce a measurable signal, and the distance of the aforesaid border from one end of the immunochromatograph is determined, in order to quantify the amount of target substance in the test sample.

Another example of an assay employing a test strip having enzyme-labeled immunochemical binding substance present thereon is found in U.S. Patent No. 4,446,232. When used for the determination of antigen, the test strip comprises a first zone containing antigens and enzyme-linked antibodies which are capable of

immunochemcially reacting with the antigens and a second zone containing a substance capable of undergoing a color forming reaction with the enzyme label linked to the antibodies, thereby to indicate the presence of the antibodies in the second zone. The antibodies are diffusively bound in the first zone, such that they will pass from the first zone to the second zone upon reaction with antigens from the test sample migrating through the first zone, but will not diffuse through the first zone in the absence of migrating antigens. In practice, the test strip is contacted with a test sample suspected of containing the target antigen for a time sufficient for the test sample to migrate along the test strip, and the presence or absence of any color change is observed as an indication of the presence or absence of the target antigen in the test sample. It is disclosed that this assay may be adapted for quantitative determinations by providing it with multiple, separate regions, each containing a different amount of immobilized reference antigen, the position of the color change being dependent on the concentration of the antigen in the test sample.

Although the test strips proposed heretofore for implementing the ELISA technique may provide a rather uncomplicated and relatively rapid procedure for the qualitative and/or quantitative determination of immunochemical binding substances, these prior art assays do not enable the determination of the target substance in excess of a predetermined amount, or the quantitative measurement of any excess of the target substance beyond the predetermined amount.

SUMMARY OF THE INVENTION

The present invention provides a simple and convenient method and test kit for determining the presence of a bindable substances in a test sample in excess of a predetermined amount and the quantitative measurement of any such excess. In carrying out the method, there is provided an array of complementary binding substance which is capable of binding to the bindable substance and having a predetermined binding capacity for the bindable substance. The test sample and the array of complementary binding substances are contacted for a time sufficient to allow binding of bindable substance in the test sample to the complementary binding substance. Thereafter, a carrier medium containing a given amount of labeled bindable substance is contacted with the array of complementary binding substance. The given amount of labeled bindable substance is such that, when added to the predetermined amount of bindable substance being determined, the binding capacity of the complementary binding substance is substantially filled. The absence or significant presence of unbound labeled bindable substance is then determined to differentiate whether or not the bindable substance is present in the test sample in excess of the predetermined amount. Since the binding capacity of complementary binding substance would be substantially filled by the sum total of the predetermined amount of bindable substance being determined (if present in the test sample) and the given amount of labeled bindable substance that is brought into contact with the array of complementary binding substance, the significance presence of unbound labeled bindable substance indicates that bindable

substance was present in the solution in excess of the predetermined amount. The amount of unbound labeled bindable substance can also be determined quantitatively to give a measurement of the degree to which the bindable substance in the test sample exceeds the predetermined amount. The array of the present invention is described as a homogeneous array, in that no separation of bound and free specific binding pair substance is required.

The test kit of the present invention comprises the substances and devices necessary to perform the above-described method. The test kit includes: 1) an array of complementary binding substance on a support, the array having a predetermined binding capacity for the bindable substance being determined; and 2) labeled bindable substance in an amount sufficient, when added to the predetermined amount, to substantially fill the binding capacity of the complementary binding substance.

The method and test kit of the present invention provides a simple, rapid and reliable method for determining chemical inbalances in various biological and other fluids. The present invention may be used to particular advantage in medical diagnosis and in detecting drugs in relatively low concentrations in body fluids.

DESCRIPTION OF THE INVENTION

The present invention is directed to the qualitative and quantitative determination of one substance of a specific binding pair, consisting of a bindable substance and its complementary binding substance. In the description of the invention that

follows, the binding pair substance being determined is referred to as the bindable substance, although in practice, either of the binding pair substances could be determined using the method and test kit of the present invention.

The term "bindable substance" refers to any substance which reacts with a complementary binding substance based on the mutual specific binding affinity between the two substances. The term "bindable substance", as used herein, includes, but is not limited to proteins, hormones, both polypeptides and steroids, carbohydrates and glycoproteins. Representative examples of specific binding pairs are antigens and their antibodies, haptens and their antibodies, hormones and their receptors, vitamins and their receptors and toxins and their receptors, determinations of all of which are within the scope of the present invention.

The term "complementary binding substance" refers to the specific binding partner of the bindable substance being determined. The complementary binding substances used in the practice of this invention are generally underivitized, which means that they are not bound to, or otherwise conjugated with, any other chemical moiety. The complementary binding substance is provided in the form of an array on a suitable support. The support can be in the form of a glass or plastic capillary, a paper strip, polymer beads, a column packed with a suitable matrix, or the like. There are many methods well known to those skilled in the art for affixing a complementary binding substances to such supports. Such methods employ, for example, covalent bonding as well as other types of affixation,

such as absorption. Any method may be employed that avoids significant diffusion of the bound substance. The complementary binding substance is preferably applied to the support as a one-dimensional lattice, by which is meant a linear array of binding sites such that target substance binding and saturation begins at one end and proceeds uniformly to the other. This may be achieved by arranging the binding substance on a three dimensional support whose length is significantly greater than width, height or cross sectional area. Alternatively, the binding component may be affixed to a two dimensional support whose length is significantly greater than its width.

The "predetermined amount" employed as the standard in carrying out the present invention may vary depending on the particular type of bindable substance in question and the nature of the assay being conducted. In the case of biological fluids, for example, there exists established levels of various components considered to be the normal or average amounts, which are commonly referred to as "clinical norms". In applying the present invention as an immunoassay, the predetermined amount of bindable substances to be determined would generally be the clinical norm.

By way of example, the following table presents the clinical norm for some of the various components of human serum:

Component	Normal Value
α-1 Antitrypsin	210-510 mg/dl
Ceruloplasmin	20-35 mg/dl
Cholesterol	150-280 mg/dl
Creatinine	0.8-1.2 mg/dl

Component	Normal Value
Folate	5-21 ng/ml
Gastrin	30-200 pg/ml
IgG	720-1500 mg/dl
IgA	90-325 mg/dl
IgM	45-150 mg/dl
Lipids	450-100 mg/dl
Albumin Proteins	4.0-5.0 g/dl
Globulin Proteins	2.0-3.0 g/dl
Quinidine	4-6 mcg/ml
Serotonin	0.1-0.32 mcg/ml
Thyroxine (total)	4-11 mcg/dl
Thyroxine (free)	0.8-2.4 ng/dl
Uric Acid	3.0-7.0 mg/100 ml
Vitamin A	0.15-0.6 mcg/ml
Vitamin B_{12}	330-1025 pg/ml

In addition to the array of complementary binding substance, the method of the present invention involves the use of a labeled form of the bindable substance being determined, or a substance having the same binding affinity for the complementary binding substance as the bindable substance being determined. Suitable labels include radioisotopes, enzymes and chromophoric substances, the latter including dyes which absorb light in the U.V. or visible region of the electromagnetic spectrum, and fluroescent or phosphorescent substances. Appropriate methods for linking of any of the aforesaid labels to a bindable substance of the type described above are well known to those skilled in the art. An important requirement of the method of labeling the bindable substance is that it not sterically hinder or interfere with the reactive sites of either component of the specific binding pair. Radioactive or phosphorescent labels rarely interferes with the stereochemical properties of chemical substances to which they are linked. In a system in which the target bindable substance is antigen or

hapten, with the array of complementary binding substance consisting of antibody to said antigen or hapten, the labeled bindable substance may consist of antigen covalently linked to an enzyme. Of course, in this embodiment, the enzyme must be linked to the antigen or hapten in such a way that the affinity of the antigen or hapten for the antibody is not impaired, or otherwise diminished. Enzymes that have exhibits enzymatic activity after in conjugated form include, catalases, perioxidases, glucuronidases, glucosidases, galactosidases, urease and oxidoreductaces, such as glucose oxidase and galactose oxidase.

An important aspect of the present invention is that a known relationship exists between the value of the predetermined amount (i.e. the clinical norm in the case of an immunoassay) of the bindable substance being determined, the amount of label, and the number of available binding sites of the complementary binding substance on the array. Since the bindable substance and the label are the same substance, or are substances having substantially the same binding affinity for the complementary binding substance on the array, any bindable substance present in the test sample competes equally with the labeled bindable substance for the available binding sites on the complementary binding substance. Because the array of complementary binding substance has a known binding capacity for the bindable substance, which is substantially filled by the clinical norm of bindable substance and the given amount of labeled binding substance used in carrying out the method, the presence of bindable substance in the test sample in excess of the clinical norm may be readily determined. Thus, if more than the clinical

norm of bindable substance is present in the test sample, there will not be sufficient binding sites on the array to accommodate all of the unlabeled and labeled bindable substance and and an excess, or "spillover", of both unlabeled and labeled bindable substance will result. Since the unlabaled and labeled bindable substance are competing for the available binding sites on the array of complementary binding substance with equal affinity, the spillover will virtually always contain labeled bindable substance, since, as noted above, the unlabeled and labeled bindable substance will be bound in proportion to the relative amount of each that is contacted with the array of complementary binding substance. Accordingly, the significant presence of unbound labeled bindable substance in the spillover differentiates whether or not the bindable substance in the test sample exceeds the established norm. As used herein, the expression "significant presence" refers to an amount which is two-three-times background noise, or error level in the test system.

In practice, a test sample believed to contain bindable substance to be determined is contacted with the array of complementary binding substance for a time sufficient to allow binding of the bindable substance to the complementary binding substances to occur. The available binding sites on the complementary binding substance are filled to the extent of the bindable substance present in the test sample. The array is then contacted with labeled bindable substances in a carrier medium. Suitable carrier mediums include aqueous or other polar solvents, such as ether or alcohol for biological systems. Non-polar solvents may

be used in other systems where appropriate. The carrier medium may be buffered to the desired pH range, and a detergent may be added to reduce non-specific binding. If the test sample contained the clinical norm of bindable substance, then the labeled bindable substance will substantially fill the remaining available binding sites of the complementary binding substance on the array.

Instead of the sequential procedure just described, the labeled bindable substance may be incorporated in the test sample for contact with the array of complementary binding substance simultanesouly with the bindable substance being determined. According to the this procedure, the labeled and unlabeled bindable substances compete directly for available binding sites on the array. In this procedure the test sample serves as the carrier medium.

In performing the method of the present invention for the determination of a particular antigen in a sample of human serum, the number of sites provided on the array of conjugate antibody substantially equals the amount of antigen corresponding to the clinical norm and the amount of labeled antigen which is added in carrying out the method. Since the number of available binding sites on the array is known, and the clinical norm of the antigen in question is also known, the amount of labeled bindable substance employed may be varied to achieve the desired substantial filling of the array. For example, if the clinical norm for a certain antigen fills 60 binding sites on an array of antibody, then an array having, for example, 120 available binding sites may be provided and used in

conjunction with an amount of labeled antigen which will substantially fill the remaining 60 binding sites. Of course, if it is desired to change the number of binding sites, one must only change the amount of label used to still achieve the intended result. After the array is contacted with both the test sample and the labeled antigen (either sequentially or simultaneously, as noted above), if more than 60 units of antigen are present in the test sample, then all of the labeled antigen will not be bound to the array. The absence or significant presence of label remaining in the test sample (or carrier medium) after contact with the array thus indicates whether the particular antigen in question is present in the serum in excess of the clinical norm.

The present method is adaptable for use in a physician's office or at home, in the form of a simple test kit. The test kit includes a suitable support to which is affixed an array of complementary binding substance having a predetermined number of binding sites, along with a predetermined amount of labeled bindable substance. The amount of labeled bindable substance would depend on the clinical norm of the substance being tested for and the number of sites on the array. If such a test kit employed enzyme as the label, a visible signal would be produced in the spillover thus obviating specialized detection equipment. Such an assay could be performed on body fluids, such as saliva or urine by relatively untrained personnel.

In accordance with the present invention, it is also possible to quantitatively measure the amount by which bindable substance in a test sample exceeds an

established norm. When the above-described procedure is performed on a test sample of serum containing more than the clinical norm of a particular antigen, the effluent obtained after contacting the test sample or carrier medium with the array contains unbound labeled and unlabeled antigen. The amount of unbound labeled antigen in the effluent may then be measured. In the case of an enzyme label, the enzymatic activity of the effluent may be determined, for example, by measuring color intensity in a colorimeter. If a radioactive label is used, the radioactivity of the effluent may be measured. Once the amount of labeled antigen in the effluent test solution or carrier medium is determined, the amount of antigen initially present in the sample solution can be easily calculated, since binding of the labeled and unlabeled antigen occurs proportionally to the concentration of labeled and unlabeled antigen initially present. Specific measurements may be made using a standard curve, as is well known in the art.

For example, where a column is employed containing an array of antibodies providing 300 binding sites, and the clinical norm for the antigen in the test sample occupies 80 of the binding sites, 220 units of labeled antigen are added to the test solution. Whenever more than the clinical norm of antigen is present in the test sample, a corresponding increase in the amount of labeled antigen remaining in the effluent test solution will be detectable. Table I, below, shows the theoretical amounts of labeled antigen which would be "spilled over" in the effluent when 220 units of labeled antigen are mixed with gradually increasing quantities of unlabeled test antigen and run on an array of antibody having a 300 binding site

capacity.

Units Test Antigen Added (Ag)	Total Antigen In "Spill Over" 300-(220+test Ag)	Labeled Antigen in "Spill Over" 220/(220+test ag) x spilled Ag
80	0	0
100	20	13.8
120	40	25.8
140	60	36.6
180	100	55
200	120	62.9
220	140	70
300	220	93
400	320	113
500	420	128

A plot of effluent activity (labeled antigen in "spill over") plotted against test antigen present in the sample solution (in excess of 80 units) provides a curve which is substantially linear. Once a table of this nature is prepared for a given antigen and its antibody it is thereafter only necessary to measure the enzyme activity of the labeled antigen in the "effluent" to find the amount of antigen present in the test sample in excess of the clinical norm.

In yet another embodiment of the present invention, it is contemplated that the array of complementary binding substance may be affixed to a solid support with an indicator substance present on

the solid support adjacent to the array. For example, if it were desired to test a sample of serum for an antigen component normally present in an amount of 80 units, an array of antibody may be provided having a binding capacity of, for example, 300 units of antigen. 220 units of enzyme labeled antigen would then be added to the test sample or to a separate carrier medium. Adjacent to the array of 300 sites, on a separate portion of the same solid support, there would be affixed an indicator substance, such as a substrate which produces a color change under the influence of the enzyme label. The sample solution would then be contacted with the array so as to fill the 300 available binding sites before reaching and contacting the indicator substance. If the amount of antigen present in the sample solution were not greater than the clinical norm of 80 units, then substantially all of the antigen, both labeled and unlabeled, would be bound to the array of antibody, and none of the antigen would reach the indicator portion of the support. If, however, the test sample were to contain more than the clinical norm of 80 units of antigen, the labeled antigen would reach the indicator substance on the support and produce an visible signal, by means of a color change.

This latter embodiment of the invention enables quantitative determination of the amount by which the bindable substance in a test sample exceeds the clinical norm. Referring to the preceding example in which an indicator substance is employed, the area of the indicator substance on the solid support which undergoes reaction with the label of the immunoreactive substance relates to the amount of the substance

present in excess of the established norm. The area of the indicator substance undergoing reaction with the label bears essentially the same functional relationship to the excess as the data reported above in Table I.

It is preferred to employ a porous cellulosic material, e.g. an elongated paper strip, as the solid support in the embodiment just described, so as to permit the test sample to diffuse readily along the support by capillary action. Immunoreactive substances and indicator substances such as enzymes may be bound directly to a paper support by methods well known in the art.

The following examples further describe the manner and process of making and using the present invention and set forth the best mode currently contemplated for carrying out the invention, but are not to be construed as limiting the invention.

EXAMPLE I

This example describes the making of a test device for use in the determination of DNP and DNP-derivitized proteins, in accordance with the present invention.

Rabbit antiserum directed against DNP was prepared by immunization of rabbits with dinitrophenylated random sequence polypeptide composed of 40% glutamic acid, 30% lysine and 30% alanine. The synthetic polypeptide had 10% of its lysines conjugated with DNP. Using precipitation analysis with DNP_{10}Bovine Serum Albumin ($DNP_{10}BSA$), the titer of the antiserum with respect to DNP was determined to be 2.1 mg. antibody/ml. of serum.

Antiserum was coupled to Sepharose 4B by the sodium carbonte/Sepharose/cyanogen bromide activation method. Prior to coupling to Sepharose, the antiserum was decomplemented by adsorption with rabbit BSA anti-BSA precipitates, dialyzed against buffer (0.1 M $NaHCO_3$) and centrifuged in a microfuge. To achieve

uniform coupling all reagents were mixed rapidly and resulted in an adsorbent slurry.

Micro-columns containing the adsorbent slurry were constructed using 20 microliter disposable micro-pipettes as the body of the micro-columns. A 10 to 15 cm. length of 1.5 mm. internal diameter plastic tubing was fitted to the bottom of the micro-pipette to serve as an outflow tube. Prior to fitting the plastic tubing over the end of the micro-pipete a small piece of cotton or a fragment of tissue was inserted into the end of the plastic tubing. When fitted over the end of the micro-pipette, the tissue or cotton material butted against the bottom of the micro-pipette forming a barrier through which adsorbent could not pass. To faciliate filling of the micro-columns the micro-pipette was fitted with a funnel constructed by taking a standard laboratory plastic pipette tip and slicing the end to allow insertion of the micro-pipette.

The micro-columns were first filled with phosphate buffered saline (PBS) containing 1% bovine serum albumin and incubated for 1 hour to reduce non-specific adsorption to the sub-nanogram levels. The columns were filled by introducing an appropriate charge of the adsorbent slurry. Each column was brought to a bed volume of 20 microliters by removing excess adsorbent using a needle and syringe. The columns were 69 to 72 mm. high and could be filled with consistency to within 0.5 mm. Therefore, bed volumes could be controlled to within 0.7%.

The micro-columns were calibrated using radioactive $DNP_{1.8}BSA$. The $DNP_{1.8}BSA$, which was carefully determined by appropriate

absorbance measurements at 280 and 360 nanometers, was labeled with iodine 125 by the Iodogen method. An activity of 6 microCuries/Microgram was achieved and it was found, as result of binding to mini-columns (100 to 300 microliters), that the labeled protein was undamaged by labeling, since the appropriate amounts of labeled protein were bound when diluted with unlabeled protein.

To determine the capacity of the micro-columns, saturating levels of labeled antigen were delivered into each of seven of the micro-columns. The labeled antigen was delivered into the micro-columns in volumes of 20 through 80 microliters. After loading the columns with the labeled antigen, 5 column volumes of 1% BSA/PBS were passed through the columns. The average number of counts bound was reliable with a deviation of approximately 5%, regardless of the number of counts introduced to the column. The capacities of the columns is set forth in Table II.

Table II

sample column#	counts delivered	counts bound
1	6,500,000	570,460
2	10,000,000	610,310
3	8,500,000	585,700
4	2,000,000	620,120
5	1,200,000	605,360
6	800,000	578,200
7	650,000	592,450

The average number of counts bound was 595,660. From the specific activity of the diluted antigen, this corresponds to 460 nanograms of antigen.

EXAMPLE II

This example describes an experiment in which labeled DNP/BSA was added to the test sample for

simultaneous delivery to the test device with unlabeled DNP/BSA.

Mini-columns of 460 nanogram capacity were constructed as described in Example I. Samples containing known amounts of unlabeled DNP/BSA were mixed with 200 nanograms (260,000 counts per minute) of labeled DNP/BSA and applied to the micro-columns. After delivery of the test samples seven column volumes of BSA/PBS were passed through each column, and the effluent was collected in a counting vial and counted. The results are shown in Table III, two trials being run of each sample.

Table III

unlabeled antigen added (nanograms)	Trial 1	Trial 2
0	7024	6842
240	6785	7210
260	7320	7428
280	11960	12200
300	21406	22780
360	45500	48200
450	76635	79200
550	105149	98460

From the results of these experiments it can been seen that background counts are collected for 0,240, and 260 nanogram levels, while the collected values climb rapidly above background once the column capacity is exceeded (starting at 280 ng).

EXAMPLE III

The experiment of Example II was performed using a sequential rather simultaneous delivery of the labeled and unlabeled DNP/BSA to the test device.

The test samples were delivered to the column first in a volume of 30 microliters, followed by 20 microliters of buffer and then by 200 nanograms (260,000 counts per minute) of the labeled antigen.

The results of this experiment are set forth in Table IV.

Table IV

unlabeled antigen (nanograms)	Trial 1	Trial 2
0	5840	6540
240	7013	5923
260	7550	7450
280	27575	28360
300	51400	55290
360	130600	136400
450	266010	257380
600	264800	255600

The results of this experiment show that enhanced sensitivity can be obtained using this procedure, as the radioactivity of the effluent is substantially increased. This is because no initial dilution with unlabeled antigen occurs. It is noted that a small margin of error exists, since more counts were present in the effluent than were introduced in the case of two of the samples. However, this error may be considered experimentally acceptable.

EXAMPLE IV

This example describes a experiment employing a different calibration system which was found effective for measuring lower levels of antigen. For this experiment, rabbit antiserum was first diluted with normal rabbit serum 1:5 to lower the level of antibody on the absorbant. Calibration of the micro-column was carried out as described previously and showed that the 20 microliter columns had a total capacity for 120 nanogram of antigen. In carrying out this experiment, 60 nanograms of labeled DNP-BSA (80,000 counts per minute) were mixed with known samples and passed through the columns. The effluents were collected and counted as previously described. The results of this experiment are given in Table V.

Table V

unlabeled antigen (nanograms)	Trial 1	Trial 2
0	1290	-
20	980	-
40	1123	-
60	1230	-
80	10800	11560
100	20400	23500
140	28700	29500
200	45000	41980

Although the method and test kit of the present invention has been described with specific reference to use as an immunoassay, the inventions has other utilities as well. For example, the invention may be used for the determination of specific binding pair substances, (e.g. DDT and naturally occurring DDT-binding substances), present as hazardous

contaminants in ground water in excess of standards established by federal regulation. Thus, if the method and test kit of the present invention were used for assaying ground water for compliance with federal regulatory requirements, the predetermined amount would be the standard established by the relevant regulation.

While the method and test kit of the present invention have been described herein in terms of certain preferred embodiments, various other embodiments may be apparent to those skilled in the art. Therefore, the invention is not limited to the embodiments actually described, but is capable of variation and modification without departing from the spirit and scope of the appended claims.

What is claimed is:

1. A method for determining whether or not one of a pair of substances having mutual specific binding affinity, and consisting of a bindable substance and a complementary binding substance, is present in a test sample in excess of a predetermined amount, said method comprising:

a.) providing an array of complementary binding substance having a predetermined binding capacity for said bindable substance;

b.) contacting said array of complementary binding substance with:

(i) said test sample, for a time sufficient for any bindable substance present in said test sample to bind to said complementary binding substance; and

(ii) labeled bindable substance in a carrier medium, the amount of said labeled bindable substance being sufficient, when added to said predetermined amount, to substantially fill the binding capacity of said complementary binding substance, said array of complementary binding substance and said labeled bindable substance being in contact for a time sufficient for said labeled bindable substance to bind to said complementary binding substance; and

c.) determining the absence or significant presence of unbound labeled bindable substance to differentiate whether or not said bindable substance is present in said test sample in excess of said predetermined amount.

2. A method according to claim 1, wherein said carrier medium for said labeled bindable substance is said test sample.

3. A method according to claims 1 or 2, wherein the test sample is human serum, the bindable substance is antigen, and the complementary binding substance is antibody specific to said antigen.

4. A method according to claim 3, wherein the predetermined amount of antigen is the clinical norm for a given volume of human serum.

5. A method according to claim 3, wherein the antibody is arrayed on an elongated substrate.

6. A method according to claim 5, wherein said antibody is arrayed on the interior surface of a capillary tube.

7. A method according to claim 5, wherein said antibody is arrayed on a paper strip.

8. A method according to claim 3, wherein the array comprises polymer beads having antibody bound thereto.

9. A method according to claims 1 or 2, wherein the label is selected from the group of an enzyme, a radioisotope, or a chromophoric substance.

10. A method according to claims 1 or 2, wherein

the unbound labeled bindable substance is qualitatively determined.

11. A method according to claim 10, wherein the label is an enzyme, the test sample is collected after contact with said array of complementary binding substance and the unbound enzyme-labeled bindable substance is determined by adding to the collected test sample a substrate which changes color under the influence of said enzyme.

12. A method according to claim 10, wherein the unbound labeled bindable substance is determined by placing adjacent to said array of complementary binding substance an indicator substance responsive to the label of said labeled bindable substance, said test sample contacting said indicator substance after contacting said array of complementary binding substance, whereby the presence of any unbound labeled bindable substance in said test sample contacting said indicator substance is indicated by said indicator substance.

13. A method according to claims 1 or 2, wherein the unbound labeled bindable substance is quantitatively determined.

14. A method according to claim 13, wherein the label is an enzyme, the test sample is collected after contact with said array of complementary binding substance and the unbound enzyme-labeled bindable substance is determined by adding to the collected test

sample a substrate for said enzyme and measuring the enzyme activity in said solution.

15. A test kit for determining whether or not one of a pair of substances having mutual specific binding affinity, and consisting of a bindable substance and a complementary binding substance, is present in a test sample in excess of a predetermined amount, said test kit comprising:

a.) a complementary binding substance capable of binding to said bindable substance, said complementary binding substance being arrayed on the surface of a support for contact with said test sample, said complementary binding substance having a predetermined binding capacity for said bindable substance; and

b.) labeled bindable substance in an amount which, when added to said predetermined amount, is sufficient to substantially fill the binding capacity of said complementary binding substance.

16. A test kit according to claim 15, further comprising an indicator substance for indicating the presence of unbound labeled bindable substance in the test sample after contact with said complementary binding substance.

17. A test kit according to claim 15, wherein said complementary binding substance is antibody and the labeled bindable substance is labeled antigen having specific binding affinity for said antibody.

18. A test kit according to claim 17, wherein the label is selected from the group of an enzyme, a radioisotope or a chromaphoric substance.

19. A test kit according to claim 16, wherein the label is an enzyme and the indicator substance is a substrate which changes color under the influence of said enzyme.

20. A test kit according to claim 15, wherein said support further comprises an indicator substance responsive to the label of the labeled bindable substance, said indicator being disposed on said support adjacent to said complementary binding substance.

21. A test kit according to claim 20, wherein the label is an enzyme and the indicator substance is a substrate which changes color under the influence of said enzyme.

22. As an article of manufacture, a solid support having affixed thereto a substantially one dimensional array of underivitized antibody providing a predetermined number of antigen binding sites and, adjacent to said array of antibody, a substrate for an enzyme capable of linking to antigen having specific binding affinity for said antibody, said substrate changing color under the influence of said enzyme.

23. An article of manufacture according to claim 22, wherein the solid support is elongated and the

array of antibody and the substrate are arranged lengthwise on said solid support.

24. An article according to claim 22, wherein said support is a paper strip.

25. An article according to claim 22, wherein said support is a capillary tube, and the antibody and substrate are arranged on the interior surface of said capillary tube.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00668

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4] G01N 33/50,53,538

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/4,7,805,810 436/518,527,530,531,534,808,809,810

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [6]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US,A, 4,446,232 (LIOTTA) 1 May, 1984. See the entire document	22-25 1-21
A,P	US,A, 4,533,629 (LITMAN) 6 August 1985	
Y	US,A, 3,991,174 (GRUNDMAN) 9 November 1976, see column 2, lines 6-17	1-21
Y	US,A, 4,039,652 (ADAMS) 2 August 1977, see columns 3 and 4 and column 9 lines 62-64	1-21

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
07 May 1986	22 MAY 1986
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Robert Benson Robert Benson